SOLUBILITY AND PHYSICAL STATE OF NITROGEN GAS IN NITROGEN FIXING AZOTOBACTER CELLS

A DISSERTATION

SUBMITTED TO THE BOARD OF UNIVERSITY STUDIES OF THE JOHNS HOPKINS UNIVERSITY IN CONFORMITY WITH THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

BY

HANS LINEWEAVER

1936

Reprints from the *Journal of Biological Chemistry*, **122**, 540 (1938), and the *Journal of Bacteriology*, **35**, 501 (1938).

SOLUBILITY AND PHYSICAL STATE OF NITROGEN GAS IN NITROGEN FIXING AZOTOBACTER CELLS

A DISSERTATION

SUBMITTED TO THE BOARD OF UNIVERSITY STUDIES OF THE JOHNS HOPKINS UNIVERSITY IN CONFORMITY WITH THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

BY

HANS LINEWEAVER

1936

Reprints from the *Journal of Biological Chemistry*, **122**, 540 (1938), and the *Journal of Bacteriology*, **35**, 501 (1938).

R. UNIV.
BIBLIOTHEEK
LEIDEN

Reprinted from The Journal of Biological Chemistry
Vol. 122, No. 2, January, 1938

THE SOLUBILITY AND CHEMICAL AND PHYSICAL ABSORPTION OF NITROGEN GAS IN AZOTOBACTER CELLS

By HANS LINEWEAVER

(From the Biochemical Nitrogen Fixation Section, Fertilizer Research Division, Bureau of Chemistry and Soils, United States Department of Agriculture, Washington)

(Received for publication, October 1, 1937)

Under suitable conditions the free living, aerobic soil organism *Azotobacter* fixes nitrogen at a surprisingly rapid rate, considering that the fixation occurs at ordinary temperatures and pressures. The maximum rate of fixation by *Azotobacter vinelandii* that has been obtained in liquid cultures at 36° and 0.78 atmosphere of nitrogen pressure corresponds to a fixation of about 0.04 gm. of nitrogen per gm. of cell dry weight per hour. The rate of fixation by inorganic catalysts is about 6 gm. per gm. of catalyst per hour in the Claude process (t = 500–650°, p = 900 to 1000 atmospheres) and 0.3 to 0.4 gm. per gm. of catalyst per hour in the Haber process (t = 500–600°, p = 200 atmospheres). The products of fixation by *Azotobacter* are in good part bound up in the cell in the form of protein-like material.

The kinetics of nitrogen fixation are specifically interpretable by the Michaelis-Menten theory of enzyme action (13, 11, 10) according to the following representation

$$N_2 + E \rightleftharpoons N_2E \quad (1)$$

$$N_2E \rightarrow E + P \quad (2)$$

where P does not necessarily represent cell material, the final product of nitrogen fixation, but possibly some intermediate product formed practically irreversibly. Letting v and $V_{max.}$ represent respectively the observed and maximum velocities of the second reaction, P_{N_2} the pressure of nitrogen, and K_{N_2} (= 170

mm. of Hg) the dissociation constant of the enzyme-substrate complex, we obtain

$$v = \frac{V_{\max.} P_{N_2}}{K_{N_2} + P_{N_2}}$$

which is an accurate expression of the experimentally observed kinetics (11).

Between 20–30° the equilibrium reaction, Equation 1, has been shown to have a very low heat of reaction, $\Delta H = 0$ (± 600) calories ((2) Equations 8 and 13), and the irreversible reaction, Equation 2, a fairly large temperature coefficient ($E_a = 19{,}300$ calories; (2) p. 38) which becomes even larger at temperatures below 17° (unpublished data). These facts make it possible for the irreversible reaction to be made practically negligible by the use of low temperatures, and, theoretically at least, to ascertain the amount of nitrogen combined with a cell enzyme component such as E, by performing a vacuum extraction on nitrogen-equilibrated cell suspensions.[1] Since the nitrogen combined in this manner should yield a hyperbolic isotherm comparable with that obtained kinetically (11), we could distinguish it from the nitrogen dissolved according to Henry's law by making sufficiently exact nitrogen gas determinations at several nitrogen pressures. For the cell concentrations employed the sensitivity of the method made it possible to detect the presence of an amount of chemical adsorption equivalent to 0.01 of the calculated amount of nitrogen required to form a monomolecular layer on the surfaces of the cells, if the nitrogen uptake agreed quantitatively with the kinetic isotherm. Positive results in such an experiment would be highly important generally, from the standpoint of the mechanism of enzyme action, and specifically

[1] The maximum error that might arise due to fixation decreasing the amount of dissolved or reversibly combined nitrogen gas during the interval between equilibration and analysis has been estimated on the following basis. For a velocity constant of fixation of 10^{-4} (1/1000 that at ordinary temperatures) about 3×10^5 molecules of nitrogen would be transformed per hour per cell. While this figure is of the order of the sensitivity involved in the analytical procedure, it is to be noted that analyses were always carried out within an hour after equilibration. Only random variations occurred on duplicate analyses carried out 20 minutes apart.

because they would give the proposed mechanism of nitrogen fixation a much more definite quantitative physical significance. Negative results, on the other hand, would show that the isotherm was not due to an extensive surface adsorption, and would establish an upper limit to the number of independently acting enzyme (nitrogenase) units in an *Azotobacter* cell.

In the present experiments isotherms for the solubility of nitrogen in water, nutrient medium, medium + sucrose, and aqueous suspensions of *Azotobacter*, legume nodule bacteria, and yeast were determined over the range 0.7–25°. In addition uptake of nitrogen and argon at 0° and −183° by dried cells was determined.

Apparatus

A thermostat holding to ±0.03° at temperatures as low as 0° contained rotating double tonometers for saturation of the liquids with gases. Fig. 1 shows the modified Van Slyke-Neill extraction chamber used with the Van Slyke blood gas apparatus (14). This chamber accomodates large samples of liquid that may be recovered without contamination with reagents and permits analyses of dissolved gases at temperatures considerably below room temperature (*cf.* Shepherd (16)). Water from the thermostat was pumped through the jacket when low temperature analyses were performed. The sample and reagent pipettes were made according to Van Slyke and Neill (18).

The uptake of nitrogen by dried cells was kindly carried out by Dr. Brunauer of this laboratory, working with Dr. Emmett, and with use of their low pressure adsorption apparatus (4).

Materials and Reagents

1.5 N potassium hydroxide was made gas-free by repeated extraction *in vacuo* and transferred, without even momentary contact with air, to a storage pipette. 0.8 cc. of this solution was used for CO_2 absorption.

Gas-free hyposulfite solution was prepared according to Van Slyke ((17) p. 575). 0.8 cc. of this solution was used for O_2 absorption.

Gas Mixtures—The four gas mixtures used were 18.03 per cent nitrogen (inert gases) in oxygen, 47.90 per cent nitrogen in oxygen, air, and nitrogen containing <0.1 per cent O_2 and <0.1 per cent

argon. These standard mixtures were compressed in suitable containers.

In Tables I to IV it will be obvious which mixture was used, since the total pressure was atmospheric and therefore the partial pressures of N_2 were approximately 135, 360, 590, and 750 mm. respectively.

Azotobacter, Rhizobium, and Yeast Cells—The physical properties of the cells employed in these experiments have been described (9). The physiological properties follow. No observations on

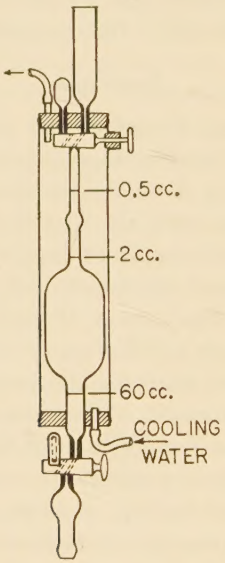

FIG. 1. Modified Van Slyke-Neill extraction chamber

the metabolic activity of the yeast and *Rhizobium* cells used in the N_2 uptake experiments were made, since their use was intended only to afford controls in regard to the uptake of nitrogen by ordinary non-fixing, one-celled plants. *Rhizobia* are active in nitrogen fixation only when growing symbiotically with their host plants.

However, in order to evaluate the results of the N_2 uptake experiments in relation to the proposed nitrogen fixation mechanism in *Azotobacter*, it was desirable to know to what extent the actual experimental *Azotobacter* material employed was capable of nitro-

gen fixation. The nitrogen fixation or growth activity of the *Azotobacter* was therefore determined, both turbidimetrically and by respiration rate increase, on diluted material. These experiments of 6 to 8 hours duration were performed on the Warburg apparatus at 31° in both free and fixed nitrogen, and at several cell concentrations (the usual values of g, the specific rate of increase in respiration rate, turbidity, etc., range from 0.15 to 0.25 for free nitrogen and 0.20 to 0.35 for fixed nitrogen; *cf.* (2, 11)).

Experiment 38—This sample showed only a low nitrogen-fixing ability. The value of g in fixed nitrogen, measured by both respiration rate and turbidity increase, was more than one-half the usual value, but in free nitrogen the g was of the order of one-tenth the usual value. This observation perhaps implies that these cells contained a low concentration of nitrogenase, the enzyme presumed to combine reversibly with N_2 gas.

Experiment 46—Comparison of the increase in respiration rate and turbidity showed that the two methods did not parallel each other. The g values determined turbidimetrically were, of course, considered the better basis for estimating the fixation activity. Values of g, so obtained, were one-fourth to one-half the usual value in either free or fixed nitrogen.

Experiment 60—Qualitatively an increase in turbidity was obtained in both free and fixed nitrogen. The order of activity, however, appeared to be more nearly like that of Experiment 38, so that here again the amount of nitrogenase per cell might possibly be expected to be low. The c.mm. of O_2 consumed per mg. of dry weight per hour was measured for this culture at both 31° and 0.7° and found, respectively, to be 600 and 1.1, each accurate to about 10 per cent. If the rate of respiration were considered to treble for each temperature increase of 10°, the value 30 would be expected at 31° instead of 600. It is therefore evident that there occurs considerable deviation from the situation existing between 20–30°, where a fairly constant temperature increment of 19,330 calories was obtained (12).

Experiment 64—Respiration rate and turbidity observations were made with both diluted, centrifuged cells and uncentrifuged cells. Typical respiration rate g values were observed for both materials whether free or fixed nitrogen was used. The turbidity g values were, however, about one-half the respiration rate g

values for the centrifuged cells, but only about 20 per cent less for the uncentrifuged cells. It appears that although a fairly satisfactory amount of nitrogenase per cell was assured by the rate studies, the supercentrifugation and resuspension apparently did injure the cells to a certain extent, in some general manner.

As previously mentioned, numerous factors affect the activity of *Azotobacter* and it is not surprising that cultures obtained by the 17 liter culture technique should yield values of g different from those obtained when the usual 100 cc. culture method is used (2). The most probable explanation for the fact that the respiration and turbidity g values do not agree is that the method of culture produces a mixed population, consisting of cells respiring only, cells respiring and reproducing normally, and cells respiring and reproducing subnormally. When placed under the conditions employed in the Warburg technique, the distribution of the population no doubt gradually changes subject to the new conditions. These observations of low rates of fixation, which, of course, fail to supply evidence that the amount of nitrogenase per cell was as high as that usually encountered in the kinetic studies, do not necessarily *prove* that the nitrogenase content of the cells was low. This readily follows when one considers that the rate of growth may be low due to any one of a large number of steps in the growth process being affected. In fact, the fixation step has been shown, generally, to be relatively less sensitive to factors such as culture condition, inhibitors, etc., than the respiration and growth processes (2). Hence, it is not proper to conclude that cells temporarily reproducing only slowly or not at all are deficient in nitrogenase. This position is possibly further supported by the reported stability of nitrogen-fixing cell-free extracts of *Azotobacter* (Bach and coworkers (1)). Only in Experiment 38 where a considerable difference between the free and fixed nitrogen g values was found does the conclusion that the cells were low in nitrogenase seem at all warranted.

The view that the *Azotobacter* cells obtained by the 17 liter culture technique contained 50 to 100 per cent of the amount of nitrogenase encountered in the previous kinetic studies will be adopted, although a certain amount of caution is admittedly necessary in drawing far reaching conclusions.

Saturation of Liquids with Gas Mixtures

The experimental liquid, generally about a 40 cc. sample, was distributed between the 50 and 500 cc. tonometers in proportionate amounts. The double tonometer was then evacuated to about 25 mm. of Hg, filled with a definite gas mixture, rotated for 10 to 20 minutes to remove traces of air from the liquid, reevacuated, and refilled with the gas mixture. Two evacuations and refillings sufficed to prevent variations in the composition of the gas phase that would affect the results outside the error of the analytical solubility measurements. Alteration of nitrogen pressure for any of the gas mixtures due to different solubilities of nitrogen and oxygen was less than 0.5 mm. of Hg. The pressure in the tonometers was brought to atmospheric by opening the stop-cock at intervals and allowing the pressure to fall to atmospheric. The tonometers were rotated at the rate of about 15 R.P.M., for not less than 3 hours. This time was adequate for the attainment of equilibrium.

Analysis of Liquids

The general features of the procedures for determination of dissolved gases described by Van Slyke and coworkers (14, 17, 18) were employed. The pipettes used to transfer the liquid to be analyzed from the equilibration tonometers to the extraction chamber held 14.37 cc. at 25°. The small accessory chamber (Fig. 1) was used to store extracted gas while subsequent extractions were being made. After complete extraction thus attainable the liquid could be recovered and the chamber even rinsed before measurement of the gas. Reagents were generally introduced through the bottom stop-cock. The analysis temperature was within 5° of the saturation temperature in all cases.

Calculations

The N_2 content of the liquid calculated as volume per cent of nitrogen was obtained by multiplying the nitrogen pressure by

$$\frac{100\,a\,(A + b)}{AS\,760(1 + 0.00384t)}$$

where the volume to which the gas was brought for measurement a = 2.811 or 0.446 cc., the volume of the extraction chamber

A = 55.7 cc., the volume of the storage chamber b = 1.3 cc., and the volume of the sample S = 14.37 cc. All pipette and chamber volumes were determined at 25°; however, since the gas analyses were always made at a temperature near that at which the sample was transferred, the same volumes could be used to calculate the factor for all temperatures.

The following symbols and definitions will be used throughout this paper.

α = cc. of N_2 (0°, 760 mm.) dissolved per cc. of liquid under 760 mm. of N_2 pressure.

α° = cc. of N_2 (0°, 760 mm.) dissolved per gm. of water in the liquid under 760 mm. of N_2 pressure. The values of α° used in the calculations indicated below were chosen from Tables I and III according to the temperature and suspending medium employed (see also foot-note ||, Table IV).

$\alpha_{\text{wet cells}}$ = cc. of N_2 (0°, 760 mm.) taken up by 1 cc. of wet cells under 760 mm. of N_2 pressure.

$\alpha_{\text{dry cells}}$ = cc. of N_2 (0°, 760 mm.) taken up by 1 gm. of suspended cells (dry weight) under 760 mm. of N_2 pressure.

The solubility coefficients, α and α°, were calculated by the equations,

$$\alpha = \frac{760\,[N_2]}{100\,p}$$

$$\alpha^\circ = \alpha C_{H_2O}$$

where $[N_2]$ represents the volume per cent of N_2 found in the liquid, p the pressure of nitrogen in mm. of mercury, and C_{H_2O} the gm. of water per cc. of liquid (Table I (9)).

$[N_2]_{\text{subscript}}$ = nitrogen gas content for the material denoted by the subscript either calculated from the C_{H_2O} and the appropriate α° or actually measured. The units are cc. of N_2 per cc. of liquid material or cc. of N_2 per gm. of dry material unless otherwise stated.

Cell volume = cell volume in cc. per cc.

The N_2 taken up by the cells including internal water was arrived at as follows:

$$[N_2]_{\text{centrifugable medium}} = \alpha^\circ(1 - \text{cell volume})C_{H_2O}(p/760)$$

$$\alpha_{\text{wet cells}} = \frac{([N_2]_{\text{total}} - [N_2]_{\text{centrifugable medium}})760}{(\text{cell volume})p}$$

The N_2 taken up by the dry or solid cell material was arrived at as follows:

$$[N_2]_{\text{total medium}}^{*} = \alpha^{\circ}_{\text{medium}}\,(p/760)\,C_{H_2O}$$

$$\alpha_{\text{dry cells}} = \frac{([N_2]_{\text{total}} - [N_2]_{\text{total medium}})\,760}{(\text{dry weight})\,p}$$

* Total medium means that all the water, both inside and outside the cells, is included.

DISCUSSION

The results are presented in Tables I and IV and Fig. 2. The record numbers in Table IV refer to those in reference (9), where detailed physical characteristics of the experimental material are recorded. The values reported in Tables II and IV are the average of duplicate analyses unless otherwise stated.

The discrepancies between the observed and calculated volume per cent values for the lower nitrogen pressures in Tables II and III may be partly accounted for by the variable rare gas (argon) content of the gas mixtures. The deviations for air compared to pure nitrogen estimated from the argon content of air and the argon α values of Estreicher (7) and Lannung (8) are approximately 0.016, 0.022, and 0.025 compared to the observed values of 0.023, 0.033, and 0.032 respectively for the three temperatures (see Tables I to IV).

The observed N_2 content values reported in Table IV have been corrected by subtracting the error found in Table II, corresponding to the N_2 pressure employed. In the experiments at 6.8° and 25° the error to be subtracted was estimated by the approximate relation,

$$\text{Error } t^{\circ} \approx (\text{error } 0.7^{\circ}) \times \frac{\alpha_{t^{\circ}}}{\alpha_{0.7^{\circ}}}$$

The water contents reported at 25° (Table I (9)) were always corrected to the experimental temperature by means of the water temperature coefficient of expansion, when used to calculate the values reported in Table IV.

Solubility of N_2 in Liquids and Cells Suspended in Liquids—The values of $\alpha_{\text{wet cells}}$ (Table IV), calculated as previously described, represent solubility coefficients that would be obtained if it were possible to work with 100 per cent cells. *The coefficient for Azoto-*

bacter at 0.7° was found to vary between 0.0203 and 0.0223 with an average value of 0.0213 for three different *Azotobacter* cul-

TABLE I

Solubility of Nitrogen in Water and in Medium at 0.7°

Liquid	P_{N_2}	N_2 content	H_2O content	α	α°
	mm. Hg	*vol. per cent*	*gm. per cc.*	*cc. per cc.*	*cc. per gm. H_2O*
Water	750	2.370	1.000	0.02401	0.02401
	750	2.373	1.000	2403	2403
	750	2.369	1.000	2400	2400
	750	2.371	1.000	2402	2402
	750	2.373	1.000	2403	2403
	750	2.378	1.000	2409	2409
	749	2.376	1.000	2409	2409
	749	2.378	1.000	2411	2411
Average				0.02405	0.02405
Medium	755	2.330	0.999	0.02348	0.02350
	755	2.322	0.999	2341	2343
	744	2.288	0.999	2338	2340
	744	2.298	0.999	2348	2350
	744	2.311	0.999	2362	2364
Average				0.02347	0.02349
Medium + 1% sucrose	755	2.287	0.993	0.02304	0.02320
	755	2.289	0.993	2313	2329
	754	2.276	0.993	2296	2312
	754	2.289	0.993	2309	2325
	754	2.248	0.993	2297	2313
	741	2.230	0.993	2288	2304
	741	2.258	0.993	2316	2332
Average				0.02303	0.02309
Medium + 0.2% sucrose	744	2.282	0.998	0.02332	0.02337
	744	2.298	0.998	2347	2352
Average				0.02340	0.02344

tures. At 6.8° the value of 0.0212 was obtained. The values are 93 and 106 per cent of the solubilities in the respective suspending

TABLE II

Nitrogen Pressure Isotherm in Water and in Medium + 1 Per Cent Sucrose at 0.7°

Liquid	P_{N_2}	N_2 content		Error, $E = (a) - (b)$
		Observed (*a*)	Calculated* (*b*)	
	mm. Hg	*vol. per cent*	*vol. per cent*	
H_2O	749	2.377	2.371	+0.006
Medium + 1% sucrose	741	2.244	2.246	−0.002
H_2O	592	1.906†	1.874	+0.032
Medium + 1% sucrose	586	1.818	1.786	+0.032
H_2O	359	1.158	1.137	+0.021
Medium + 1% sucrose	355	1.093	1.076	+0.017
H_2O	135	0.440	0.427	+0.013
Medium + 1% sucrose	134	0.426	0.406	+0.020

* Calculated from the appropriate α value in Table I.

† Single analysis.

TABLE III

Solubility of Nitrogen in Water at 25° and 6.8°

Temperature	P_{N_2}	N_2 content		H_2O content	α*	α°*	Error, $E = (a) - (b)$
		Observed (*a*)	Calculated (*b*)				
°C.	*mm. Hg*	*vol. per cent*	*vol. per cent*	*gm. per cc.*	*cc. per cc.*	*cc. per gm. H_2O*	
25.0	732	1.413	1.418	0.997	0.01468	0.01472	−0.005
25.0	734	1.425	1.422	0.997	1476	1480	+0.003
25.0	734	1.423	1.422	0.997	1474	1478	+0.001
Average					0.01473	0.01477	
25.0	579	1.144	1.122	0.997	0.01492	0.01496	+0.022
25.0	579	1.146	1.122	0.997	1495	1499	+0.024
25.0	350	0.697	0.678	0.997			+0.019
25.0	350	0.698	0.678	0.997			+0.020
25.0	350	0.687	0.678	0.997			+0.009
6.8	747	2.014		1.000	0.02050	0.02050	
6.8	747	1.996		1.000	2032	2032	
6.8	590	1.628	1.585	1.000	0.02098	0.02098	+0.043
6.8	590	1.608	1.585	1.000	2073	2073	+0.023
6.8	136	0.390	0.366	1.000			+0.024
6.8	135	0.384	0.363	1.000			+0.021

* α and α° values are reported only for essentially pure N_2 and for air. The other gas mixtures are arbitrary so that the α values have no general interest.

TABLE IV

Nitrogen Taken Up by Suspensions of Azotobacter, Legume Bacteria, and Yeast

Record No.	P_{N_2}	$[N_2]_{total}$ = N_2 content		α = N_2 solubility coefficient in suspension	$\alpha_{wet\ cells}$ = cc. N_2 taken up by 1 cc. wet cells per atmosphere N_2 pressure	$\alpha_{dry\ cells}$ = cc. N_2 taken up by 1 gm. dry cells per atmosphere N_2 pressure
		Observed	Corrected			
Azotobacter; uptake at 0.7°						
	mm. Hg	*vol. per cent*	*vol. per cent*	*cc. per cc. liquid*	*cc. per cc. wet cells*	*cc. per gm. dry cells*
46	752	2.197*	2.197†	0.02220 ± 0.0001‡	0.0203 ± 0.0003‡	0.005 ± 0.002‡
	594	1.765*	1.733	2219 1	202 4	5 2
	360	1.083	1.051	2220 2	203 7	5 3
	136	0.429	0.397	2220 6	202 19	5 8
Weighted average§				0.02220 ± 0.00003	0.0203 ± 0.0001	0.0053 ± 0.0007
60	749	2.222*	2.222	0.02256 ± 0.0001	0.0216 ± 0.0003	0.009 ± 0.001
	359	1.083	1.051	2226 2	207 6	5 3
	135	0.414	0.397	2236 6	211 17	6 7
Weighted average§				0.02245 ± 0.00006	0.0213 ± 0.0002	0.0083 ± 0.0007
64	752	2.257	2.257	0.02281 ± 0.0001	0.0223 ± 0.0003	0.012 ± 0.001
	594	1.799	1.767	2261 1	217 4	9 2
	360	1.113	1.094	2310 2	233 7	15 3
	136	0.426	0.409	2287 6	225 19	12 7
Weighted average§				0.02279 ± 0.00003	0.0223 ± 0.0001	0.0116 ± 0.0005

Azotobacter; uptake‖ at 6.8°									
38	740	1.952	1.952	0.02004	± 0.0001	0.0208	± 0.0006	0.016	± 0.002
	585	1.571	1.544	2005	1	209	7	16	3
	355	0.970*	0.954	2042	2	228	12	24	5
	134	0.370	0.356	2020	6	211	31	19	13
Weighted average§				0.02012	± 0.00003	0.0212	± 0.0002	0.0173	± 0.001
Heat-inactivated *Azotobacter;* uptake‖ at 25°									
24-1	734	1.384*	1.384	0.01433	± 0.0001	0.013	± 0.004	0.004	± 0.02
24-2	734	1.372*	1.372	1421	1	11	2	−0.005	1
24-2	357	0.706*	0.692	1472	2	21	5	0.046	2
24-3	734	1.410	1.410	1460	1	17	1	22	05
Weighted average§						0.015	± 0.0005		

* Single analysis.

† The limits of error assigned for this column are ±0.010 volume per cent.

‡ The indicated experimental error for $\alpha_{\text{wet cells}}$, $\alpha_{\text{dry cells}}$, and α were obtained from the error of ±0.010 volume per cent for the direct observations by use of the following factors: for $\alpha_{\text{wet cells}}$, $\pm 0.0001 \times 760/P_{N_2} \times$ (cell volume); for $\alpha_{\text{dry cells}}$, $\pm 0.0001 \times 760/P_{N_2} \times$ (dry weight); for α, $\pm 0.0001 \times 760/P_{N_2}$. Errors other than the solubility analytical error are considered to be included in this figure.

§ The reciprocal of the limit of error was arbitrarily assigned as the weighting of the values to be averaged. The indicated errors of the averages were likewise arbitrarily estimated by the relation $0.6745 \sqrt{\Sigma w v^2 / \Sigma w(n-1)}$ where w, v, and n respectively represent the weighting, deviation of a single observation from the mean, and the number of observations. In some cases in which the deviations were apparently unduly small a higher error has been assigned.

‖ These samples of *Azotobacter* were diluted with medium (no sucrose). The α° employed in making the calculations were obtained by multiplying $\alpha^\circ_{H_2O}$ ($t°$) by the ratio, $\alpha^\circ_{\text{medium}}/\alpha^\circ_{H_2O}(0.7°)$; *i.e.*, $\alpha^\circ_{\text{medium}}$ (6.8°) = 0.02041 (0.02349/0.02405) = 0.01994, and $\alpha^\circ_{H_2O}$ (25°) = 0.01477 (0.02349/0.02405) = 0.01442.

TABLE IV—*Concluded*

Record No.	P_{N_2}	$[N_2]_{total}$ = N_2 content		α = N_2 solubility coefficient in suspension	$\alpha_{wet\ cells}$ = cc. N_2 taken up by 1 cc. wet cells per atmosphere N_2 pressure	$\alpha_{dry\ cells}$ = cc. N_2 taken up by 1 gm. dry cells per atmosphere N_2 pressure
		Observed	Corrected			
Legume bacteria; uptake at 0.7°						
	mm. Hg	*vol. per cent*	*vol. per cent*	*cc. per cc. liquid*	*cc. per cc. wet cells*	*cc. per gm. dry cells*
56	740	2.285	2.285	0.02345 ± 0.0001	0.0237 ± 0.0004	0.019 ± 0.001
	585	1.840	1.808	2350 1	238 5	19 1
	355	1.115	1.096	2348 2	236 9	18 2
	134	0.442	0.425	2410 6	261 23	26 7
Weighted average§				0.02352 ± 0.00003	0.0239 ± 0.0002	0.0190 ± 0.0004
Yeast; uptake at 0.7°						
42-1	590	1.776*	1.751¶	0.02257 ± 0.0001	0.0143 ± 0.0009	−0.0330 ± 0.004
42-2	590	1.721*	1.702¶	2192 1	170 4	−0.0081 2
42-3	590	1.530	1.525¶	1965 1	166 2	−0.0151 1
43-1	578	1.767	1.735	2236 1	175 7	−0.0142 3
43-2	578	1.648	1.616	2082 1	170 3	−0.0133 1
43-3	578	1.432	1.400	1804 1	170 2	−0.0122 1
Weighted average§					0.0168 ± 0.00006	−0.0141 ± 0.0003

¶ These suspensions were made with medium and hence have been corrected by the factor $+(2.405 - 2.347)(1 - \text{cell volume})P/760$ to make them comparable with the water-diluted cells, as well as by the factor −0.032.

liquids and are 106 and 123 per cent of the value that might be expected on the basis of the water contents. *The coefficient for legume bacteria at 0.7°* was found to be 0.0239 or 102 per cent of the solubility in the suspending medium, and 121 per cent of the value expected on the basis of the water contents. *The coefficient for a sample of Fleischmann's yeast at 0.7°* was found to be 0.0168 or 70 per cent of the solubility (0.02405) in water and 82 per cent of the value expected on the basis of the water content.

The linear relation between α and cell volume found for the yeast (compare that reported by Van Slyke, Dillon, and Margaria (17) for erythrocytes in plasma) may be expressed by the equation,

$$\alpha = 0.02385 - 0.00715 \times (\text{per cent cell volume})$$

Such a relationship was not demonstrated for *Azotobacter* and legume bacteria although, obviously, it was assumed in the calculation of $\alpha_{\text{wet cells}}$. It is to be noted that the high viscosity of cell suspensions of these two organisms practically prohibits solubility determination above 35 to 40 per cent cell volume. *The values of the coefficient* $\alpha_{\text{dry cells}}$, obtained for the *Azotobacter* suspensions at 0.7° are 0.005, 0.008, and 0.012; at 6.8° the value 0.017 was obtained. The legume bacteria yielded the value 0.019 and yeast the negative value -0.141. Van Slyke, Dillon, and Margaria (17) obtained comparable coefficients of 0.017 and 0.019 cc. per gm. of dissolved hemoglobin at 38° and 25° respectively.

The variation in the values of both $\alpha_{\text{wet cells}}$ and $\alpha_{\text{dry cells}}$ for *Azotobacter* and the change in sign with yeast may be attributed to physiological variations. Such variations would be of the nature of variable internal salt concentration, amount of "bound" water, and different proportions of cell constituents.

Absence of Measurable Reversible Chemical Combination or Physical Adsorption of Nitrogen by Suspended Cells—The N_2 dissolved by suspensions of both *Azotobacter vinelandii* and the legume bacteria, *Rhizobium meliloti*, at 0.7° and over the pressure range from 0.18 to 1 atmosphere, was found to be directly proportional to the N_2 pressure, in accordance with Henry's law (Table IV). There is no tendency for N_2 content or α values to be disproportionately high at low N_2 pressures, as would be

expected if adsorption as well as solubility of N_2 occurred. The following equation represents the proposition being tested.

$$[N_2]_{\text{content}} = kP_{N_2} + \frac{k'P_{N_2}}{K_{N_2} + P_{N_2}}$$

Volume of N_2 taken up — Solubility — Adsorption or chemical combination

The data are therefore valid in eliminating the second function on the right side of the equation, within the limits of the technique, if K_{N_2} has the value exhibited for the fixation-nitrogen pressure isotherm obtained kinetically (about 170 mm.).

It was hoped at the start of this investigation that it would be possible to carry out such measurements on active, concentrated enzyme extracts of *Azotobacter*. However, in spite of numerous attempts and of the work of Bach and coworkers (1), only inactive extracts were obtained. No further report of this work is made here. Since this work was completed Roberg (15) has reported failure to confirm the work of Bach and coworkers.

The upper limit of the number of molecules of N_2 taken up per cell by adsorption can be calculated as follows: By analysis of the data in Table IV, especially graphically, it can be seen that the maximum value the hyperbolic function in the previous equation could have is about 0.04 cc. per 100 cc. of cells at high nitrogen pressures (saturation of E). For Experiments 46, 60, and 64 the average gm. of dry weight per 100 cc. is 7.8, and there are about 6×10^{11} cells per gm. of dry weight, so that the maximum number of molecules that may be taken up per cell hyperbolically is 0.04 $(6.06 \times 10^{23})/(22{,}400)$ (7.8) $6 \times 10^{11} = 2.31 \times 10^5$. Similarly, the values 4×10^5 and 0.4×10^5 are obtained for Experiments 38 and 56. These maximum values for the nitrogen that may be taken up by *Azotobacter* cells in the prescribed hyperbolic manner may be variously expressed, approximately, as: (1) 50 per cent of the nitrogen represented by the coefficient $\alpha_{\text{dry cells}}$; (2) 6 and 1 per cent respectively of the amount of nitrogen taken up by dried powdered *Azotobacter* at 0° and −183° (see later); (3) 1 per cent of the calculated number of nitrogen molecules required to form a monomolecular layer on the external surface of cells 1 to 2 μ in diameter; (4) 0.1 per cent of the non-water molecules per cell

estimated on the basis of a diameter of 2 μ, a density of 1.1, a water content of 76 per cent, and an average non-water molecular weight of 1000.

Uptake of Nitrogen and Argon by Dried Azotobacter and Yeast Cells

Fig. 2 gives the data obtained for N_2 and A taken up by dried powdered and unpowdered *Azotobacter* at 0° and −183° and for

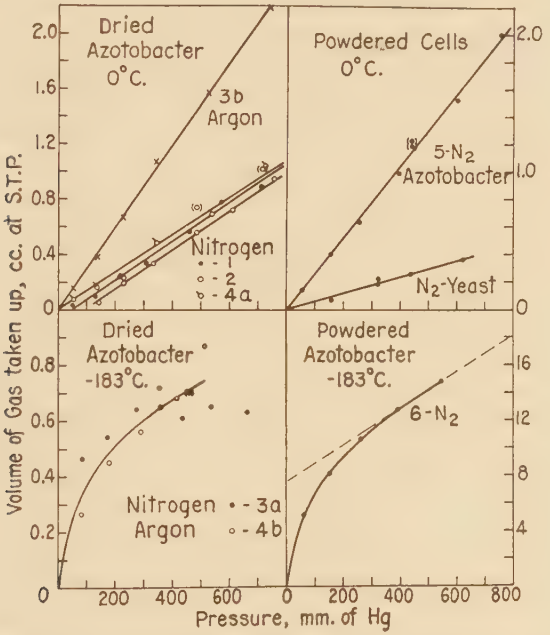

Fig. 2. The uptake of nitrogen and argon in dried powdered and unpowdered *Azotobacter* and yeast cells at 0° and −183° as a function of pressure of the gas. Nos. 1, 2, 3a, etc., give the order in which the runs were made. The points in parentheses represent values obtained either when the pressure was lowered after observation at 760 mm., or when the observation time was 18 hours. 17.4 gm. samples were used. The error is about ±0.1 cc.; the 0° data are subject to the least error. No uptake great enough to observe was obtained at 0° with unpowdered yeast.

the N_2 taken up by powdered yeast. The run numbers occurring in Fig. 2 show the order in which the experiments were carried out. It is seen, therefore, that the N_2 isotherm at 0° with un-

powdered cells is reproducible even after a run is made at −183°. The failure of some of the curves to extrapolate to zero is attributed to experimental error, which is relatively large for the amounts of gas taken up. Powdering the dried *Azotobacter* until they appeared microscopically to be individual cells, or at most groups of four or five, resulted in a doubling of the amount of N_2 taken up at 0°, but at −183° gave a 20-fold increase in N_2 uptake. Powdered yeast, reduced to individual cell size, at 0° took up one-fourth as much N_2 as powdered *Azotobacter*. Unpowdered yeast was not observed to take up any gas.

As reported by Burk (3), these observations were originally believed to result from *solubility* of these gases in the dried organisms. However, while activated adsorption is readily eliminated, since argon shows the uptake as well as nitrogen, it may still be that van der Waal's adsorption is the chief factor in the uptake. Emmett, Brunauer, and Love (6) have shown that soils take up gases by van der Waal's adsorption at 0° and −183° in the ratio 1:50, while for dried *Azotobacter* the ratio is 1:10. Thus the uptake reported here need only have a slightly different temperature coefficient than that on soils. The fact that powdering increases the uptake at 0° only 2-fold, while it increases it 20-fold at −183°, may be consistent with either interpretation. This would require only that diffusion be limiting in a greater degree at −183° than at 0° and to a greater degree for the unpowdered cells than for the powdered cells. It is interesting too that the ratio of the solubilities of argon and nitrogen is about that found for the uptake by dried cells at 0°, particularly when corrected for the fact that the dead space in the apparatus was determined with helium. Finally the concave downward isotherm obtained at −183° with powdered *Azotobacter*, which according to Emmett and Brunauer (5) may be used to calculate the surface of one cell, yielded the area 3.4 to 4.1 sq. μ. This value may be compared with the calculated surface areas 13 and 2 sq. μ of spherical cells having diameters of 2 and 1 μ respectively.

SUMMARY

Typical Henry's law solubility relations were observed for nitrogen obtainable by vacuum extraction from suspensions of *Azotobacter*, legume bacteria, and yeast cells maintained at low tempera-

tures. Hyperbolic chemical or physical adsorption, of the nature suggested by the kinetics of nitrogen fixation by *Azotobacter*, was found to be beyond the limits of measurements of the present technique. Nitrogen and argon were reversibly taken up by dried *Azotobacter* and yeast cells at 0° and −183° in readily measurable quantities.

The writer is much indebted to Dr. Dean Burk for his encouragement and suggestions during the pursuit of this work. He is also indebted to Professor J. C. W. Frazer of Johns Hopkins University for suggestions and criticisms. The valuable criticisms offered by Dr. P. H. Emmett and Dr. F. E. Allison as well as Dr. Burk in the preparation of the manuscript are greatly appreciated.

BIBLIOGRAPHY

1. Bach, A. N., Yermolieva, Z. V., and Stepanian, M. P., *Compt. rend. Acad. Sc., U. S. S. R.*, **1**, 22 (1934).
2. Burk, D., in Nord, F. F., and Weidenhagen, R., Ergebnisse der Enzymforschung, Leipsic, **3**, 23 (1934).
3. Burk, D., *Biochimia*, **2**, 312 (1937).
4. Emmett, P. H., and Brunauer, S., *J. Am. Chem. Soc.*, **56**, 35 (1934).
5. Emmett, P. H., and Brunauer, S., *J. Am. Chem. Soc.*, **59**, 1553 (1937).
6. Emmett, P. H., Brunauer, S., and Love, K., *Soil Sc.*, in press.
7. Estreicher, T., *Z. physik. Chem.*, **31**, 184 (1899).
8. Lannung, A., *J. Am. Chem. Soc.*, **52**, 68 (1930).
9. Lineweaver, H., *J. Bact.*, in press; see also Thesis, Johns Hopkins University (1936).
10. Lineweaver, H., and Burk, D., *J. Am. Chem. Soc.*, **56**, 658 (1934).
11. Lineweaver, H., Burk, D., and Deming, W. E., *J. Am. Chem. Soc.*, **56**, 225 (1934).
12. Lineweaver, H., Burk, D., and Horner, C. K., *J. Gen. Physiol.*, **15**, 497 (1932).
13. Michaelis, L., and Menten, M. L., *Biochem. Z.*, **49**, 1333 (1913).
14. Peters, J. P., and Van Slyke, D. D., Quantitative clinical chemistry, Methods, Baltimore (1932).
15. Roberg, M., *Jahrb. wissensch. Bot.*, **83**, 567 (1936).
16. Shepherd, M., *Bur. Standards J. Research*, **12**, 551 (1934).
17. Van Slyke, D. D., Dillon, R. T., and Margaria, R., *J. Biol. Chem.*, **105**, 571 (1934).
18. Van Slyke, D. D., and Neill, J. M., *J. Biol. Chem.*, **61**, 570 (1924).

Reprinted from JOURNAL OF BACTERIOLOGY
Vol. 35, No. 5, May, 1938

PHYSICAL CHARACTERISTICS OF CELLS OF AZOTOBACTER, RHIZOBIUM, AND SACCHAROMYCES[1]

HANS LINEWEAVER

Bureau of Chemistry and Soils, U. S. Department of Agriculture, Washington, D. C.

Received for publication October 25, 1937

Recent investigations concerning the mechanism of nitrogen fixation by *Azotobacter* have been concerned with kinetics (Burk, 1934; Endres, 1934a; Lineweaver, Burk and Deming, 1934), essential elements for fixation as distinguished from growth (Burk, 1934; Burk and Horner, 1935b), chemical intermediates and products of fixation (Burk and Horner, 1935a and c; Endres, 1934b and 1935; Roberg 1935; Winogradsky, 1932), and fixation *in vitro* (Bach, Yermolieva and Stepanian, 1934; Roberg, 1936). The present communication reports general physical characteristics of *Azotobacter*, and for comparison, of *Rhizobium* and *Saccharomyces* obtained during direct examination of the enzymic mechanism of nitrogen fixation previously proposed on the basis of kinetic data (Lineweaver, 1938; Lineweaver, Burk and Deming, 1934). The physical characteristics of the cells determined were the density, water content, and relation between dry weight, cell volume, and cell number.

MATERIALS AND METHODS

Azotobacter cells. *A. vinelandii*, *A. chroococcum* (Burk strain B8), and *A. beijerinckii* (Burk strain B6) were grown in large scale amounts using methods employed by members of this laboratory working with Dr. G. E. Hilbert. The bacteria were grown at 31°C. in pyrex bottles containing 17 liters of culture medium with 1 or 2 per cent sucrose. The bottles were inoculated with 100 cc. of a 2- to 4-day heavy growth of *Azotobacter*. The cul-

[1] These investigations were carried out in the Biochemical Nitrogen Fixation Section of the Fertilizer Research Division.

tures were aerated with air for 1 to 2 days and then with 1:1 oxygen-nitrogen mixture for 1 to 3 days more. The bacteria were harvested by centrifugation in a Sharples super-centrifuge, the 17 liters of culture yielding from 40 to 90 grams wet weight of bacteria.

For study, the centrifuged cells were re-suspended in culture medium, generally containing 1 per cent sucrose, and consisting of the clear liquid obtained after the following mixture had been thoroughly shaken, allowed to stand, and settle: 0.8 gram of K_2HPO_4, 0.2 gram of KH_2PO_4, 0.2 gram of NaCl, 0.2 gram of $MgSO_4 \cdot 7H_2O$, 0.1 gram of $CaSO_4 \cdot 2H_2O$, 1000 grams of H_2O. Fe as iron humate, about 0.5 mgm. per liter, and Mo as Na_2MoO_4, about 0.1 mgm. per liter, were generally added when the suspension was made. Heat-inactivated cells were prepared by rapidly heating a suspension to 90°C., and, after 5 to 15 minutes, cooling rapidly to 25°C.

Rhizobium meliloti, alfalfa strain 131, University of Wisconsin, was grown by the same technique used for *Azotobacter* and suspended in medium containing 0.2 per cent sucrose.

Baker's yeast cells were obtained from Fleishmann's Yeast Company in commercial starch-free, one pound cakes, and were suspended in medium or in distilled water.

The per cent total solids by weight, was determined by weighing samples before and after drying to constant weight at 110°C. The error was estimated to be about ± 0.15 weight per cent.

The densities of the suspensions and centrifugates were determined by weighing in a flask (11.238 cc. at 25°C.) that had a flat ground glass lip for sealing with a ground glass plate. Duplicate determinations generally agreed to within ± 0.0004 gram per cubic centimeter.

The cell volumes of the suspensions were determined as illustrated in figure 1 (cf. cell volume of blood, Peters and Van Slyke, 1932, p. 73). The cell suspensions, followed by bromobenzene, were drawn by suction into the lower opening of the tube. The rubber seal, cut from rubber tubing, is adequate since the pressure on the outside of the seal is greater than that on the inside due to the $CaCl_2$ solution. The samples were centrifuged repeatedly at

about 3500 r.p.m. and the per cent volumes read at intervals until they were found to be essentially constant; the total time required was of the order of 30 to 60 minutes.

The dry weight was determined by drying once-washed centrifuged cells to constant weight at 110°C. The error was estimated to be from ±3 to ±5 per cent.

The count, made microscopically, was accurate to ±10 per cent.

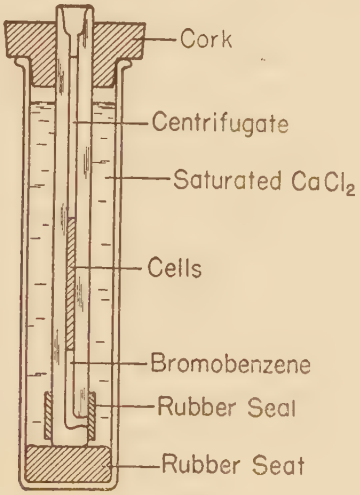

FIG. 1

CALCULATIONS

The water content and total solids by volume of the various liquids are given by the equations:

Water content (grams per cc.) = Density × per cent water by weight ÷ 100

Total solids (grams per cc.) = Density × per cent total solids by weight ÷ 100

The density of the cells and the water content of the cells are given by the following equations:

$$D_c = [D_a - D_b(1 - c.v.)]/c.v., \text{ and}$$

Water content of cells (grams per cc.) = D_c − Dry weight (grams per cc.) ÷ $c.v.$

where D_c, D_a and D_b are the densities of the cells, suspension and centrifugate respectively, and *c.v.* is the cell volume in cubic centimeter per cubic centimeter of suspension.

RESULTS AND DISCUSSION

Although an approximately constant relation between cell volume and dry weight per cubic centimeter for any one organism was observed (table 1), there was some slight variation, as indicated in figure 2. This may be attributed to a number of varying factors, such as size of inoculum, rate of aeration, and duration of growth, that are always encountered when growing microorganisms. The water contents for the cells, reported in table 2, agree with data for various bacteria and yeasts obtained by other investigators upon drying centrifuged cells at 100 to 110°C. (Buchanan and Fulmer, 1928, p. 68). The water content of bacteria generally lies between 75 and 85 per cent (Buchanan and Fulmer, 1928, table VI). The values found for *A. vinelandii* are 80, 78 and 74, for *A. chroococcum* 85, for *A. beijerinckii* 86 and for *R. meliloti* 68 per cent. The two values for the yeast, 79 and 80 per cent, although slightly higher than the average value reported in the literature, 73 per cent, fall well within the wide limits, 68 and 83 per cent.

The density and water content of one species of *Azotobacter*, *A. vinelandii*, were observed to vary between comparatively narrow limits, 1.087 to 1.106, and 74 to 80 per cent. The other two species yielded the values 1.036, 1.04 and 85 and 86 per cent, which appear to be significantly different from those of the first. The explanation for this difference may depend upon the well-known fact that different species produce different amounts of gum-like material, which may intimately surround the cells. Such material would almost certainly have both a different and more variable density and water content than the more opaque cell material.

The absolute accuracy of both the density and water content values, as well as the volume per cell and cell diameter values, depend on the accuracy of the observed centrifugal cell volume, which, in turn, depends on the extent of packing that takes

TABLE 1

Dry weight, cell volume, total solids, density, and water content of experimental material at 25°C.

MATERIAL AND RECORD NUMBER	DRY WEIGHT	PER CENT CELL VOLUME	PER CENT TOTAL SOLIDS	DENSITY		H_2O CONTENT OF LIQUID
				Liquid	Centrifugate	
	gram per cc.	*cc. per cc. × 100*	*grams per gram × 100*	*grams per cc.*	*grams per cc.*	*grams per cc.*
Medium			(0.18)	0.9981		0.996
Medium (1 per cent sucrose)			1.173	1.0021		0.990
Azotobacter†						
vin. 60	0.0816	34.0	9.08	1.0326	1.0043	0.939
vin. 64	0.0864	29.5	9.26	1.0333	1.0030	0.938
vin. 68	0.0868	39.8	10.22	1.037	1.0029	0.931
chr. 68	0.0850	56.1	9.73	1.021	1.0015	0.922
beij. 68	0.0850	57‡‡	9.94	1.026		0.921
vin. 46	0.0662	30.1				0.942*
vin. 38	0.0440	18				0.966*
vin. 24-1‡	0.0052	(2.3)††				0.992*
vin. 24-2‡	0.0103	(4.5)††				0.989*
vin. 24-3‡	0.0206	(9.0)††				0.981*
Rhizobium§						
56	0.0874	24.5	9.61	1.0274		0.929
70		2.0		1.0040	1.0020	
Yeast**						
66-1	0.0602	26.3	6.38	1.0182	0.9976	0.953
66-2	0.1701	79.8	18.40	1.0616	0.9999	0.866
42-1	0.0348	15				0.971*
42-2	0.0751	30				0.947*
42-3	0.146	60				0.899*
43-1	0.0459	19				0.965*
43-2	0.1033	40				0.930*
43-4	0.2124	80				0.865*

* Water content (46) = 0.830 (*c.v.*) + 0.990 (1 − *c.v.*) = 0.990 − 0.160 (*c.v.*); water content (38 and 24) = 0.996 − 0.166 (*c.v.*); water content (42 and 43) = 0.996 − 0.164 (*c.v.*); where *c.v.* is the cell volume in cubic centimeter per cubic centimeter.

† The diluent was medium (1 per cent sucrose), exception see footnote ‡.

‡ The diluent was medium (no sucrose).

§ The diluent was medium (0.2 per cent sucrose).

** The diluent was H_2O except for the 42 series where medium was used.

†† The cell volumes of these heat-inactivated cells were calculated by the equation: (*c.v.*) = 435 (dry weight).

‡‡ Somewhat less accurate than other figures, ±3 per cent.

place. If the cells are assumed to be rigid spheres packed horizontally, one sphere directly upon another, 47.64 per cent of the total volume would be filled with water and similarly if the spheres were packed obliquely 25.95 per cent of the space would be filled with water. The cells, however, are seldom exact

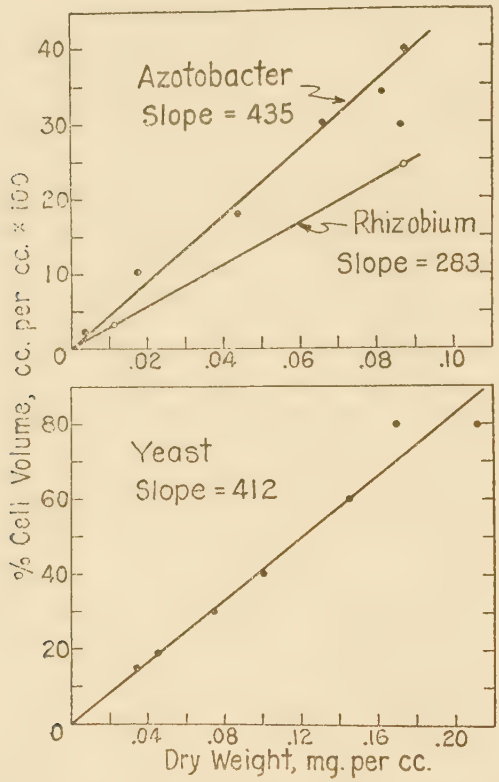

FIG. 2. THE RELATION BETWEEN CELL VOLUME, DETERMINED BY CENTRIFUGATION, AND DRY WEIGHT OF AZOTOBACTER, LEGUME BACTERIA AND YEAST CELLS

spheres and almost certainly are not rigid so that we would not expect a 25 per cent error in the water content figures on this account. Some water will, of course, always be held in the interstices by capillarity, and be a source of error. Another picture of the situation may be obtained from the observation that the volumes per cell calculated from the microscopically estimated

cell diameters were about 50 per cent of the volume per cell obtained by dividing the observed per cent cell volume by the count per 100 cc. (table 2, column 5). These diameters, however, were only about 30 per cent less than those calculated from the observed per cent cell volume (table 2). This order of agreement while not entirely satisfactory, is about as good as can be expected

TABLE 2

Size, density and water content of Azotobacter, Rhizobium and yeast cells at 25°C.

ORGANISM	COUNT	DRY WEIGHT	PER CENT CELL VOLUME	VOLUME PER CELL	CALCULATED DIAMETER	DENSITY OF CELLS	WATER CONTENT OF CELLS	
	millions per cc.	*gram per cc.*	*cc. per cc. × 100*	*c.mm. × 10^9*	*mm. × 10^3*	*grams per cc.*	*gram per cc.*	*per cent by weight*
Azotobacter								
vin. 28-1	10,000	0.0177	10.3	10	2.7			
vin. 28-2	3,200	0.0037	2.2	7	2.4			
vin. 68	30,000	0.0868	39.8	13	2.9*	1.088	0.871	80.0
chr. 68†	3,000	0.0850	56.1	187	7.1	1.036	0.883	85.0
beij. 68†	10,000	0.0850	(57)	(57)	4.8	1.04	0.892	85.8
vin. 60		0.0816	34.0			1.087	0.847	77.9
vin. 64		0.0864	29.5			1.106	0.813	73.5
Rh. meliloti								
33	33,600	0.0114	3.3	1	1.2			
70	12,000		2.0	1.7	1.5	1.10		
56		0.0874	24.5			1.106	0.749	67.6
Yeast (commercial)								
33	7,000	0.199	73.3	105	5.9			
66-1		0.0602	26.3			1.074	0.845	78.8
66-2		0.1701	79.8			1.076	0.863	80.0

* Corresponding ellipsoidal diameters, ratio 1.5 to 1, are 3.4, 2.2.

† These strains contain from 2 to 3 per cent nitrogen while the *A. vinelandii*, under these culture conditions contain 12 to 18 per cent nitrogen.

without detailed microscopic work involving shape and size of the cells, both stained and unstained, as well as counts.

SUMMARY

The relations between dry weight, cell volume, and count per cubic centimeter have been determined for three species of

Azotobacter, for *Rhizobium meliloti* and for baker's yeast. The densities and water contents of the various cells determined were: *Azotobacter vinelandii* cells 1.09 and 0.84 gram per cubic centimeter (average); *Azotobacter chroococcum* 1.04 and 0.87 gram per cubic centimeter; *Azotobacter beijerinckii* 1.04 and 0.88 gram per cubic centimeter; *Rhizobium meliloti* 1.10 and 0.75 gram per cubic centimeter; and *Saccharomyces* 1.075 and 0.85 gram per cubic centimeter.

The writer is much indebted to Dr. Dean Burk for his encouragement and suggestions during the pursuit of this work. He is also indebted to Professor J. C. W. Frazer of Johns Hopkins University for suggestions and criticisms. The valuable criticisms offered by Drs. F. E. Allison and C. A. Ludwig as well as Dr. Burk in the preparation of the manuscript are greatly appreciated.

REFERENCES

Bach, A. N., Yermolieva, Z. V., and Stepanian, M. P. 1934 Fixation de l'azote atmosphérique par l'intermédiaire d'enzymes extraites de cultures d'Azotobacter. Compt. rend. Acad. Sci. U. S. S. R., I, **1**, 22–24.

Buchanan, R. E., and Fulmer, E. I. 1928 Physiology and Biochemistry of Bacteria. Baltimore, Vol. I.

Burk, D. 1934 Azotase and Nitrogenase in Azotobacter. Ergebnisse der Enzymeforschung (Nord and Weidenhagen), Leipzig, III, 23–56.

Burk, D., and Horner, C. K. 1935a The production of ammonia by Azotobacter and its relation to the mechanism of nitrogen fixation. Trans. A, Third Intern. Cong. Soil Sci., **1**, 148–151.

Burk, D., and Horner, C. K. 1935b The specific catalytic rôle of molybdenum and vanadium in nitrogen fixation and amide utilization by azotobacter. Ibid., **1**, 152–155.

Burk, D., and Horner, C. K. 1935c Über Hydroxylamin, Hydrazin und Amide als Intermediärprodukte bei der N_2-Fixation durch Azotobacter. Naturwiss., **23**, 259–260.

Endres, G. 1934a Zur Kenntnis der stickstoffassimilierenden Bakterien I. Ann. Chem. I, **512**, 54–80.

Endres, G. 1934b Über ein Zwischenprodukt der N_2-Assimilation. Naturwiss., **22**, 662.

Endres, G. 1935 Zur Kenntnis der stickstoffassimilierenden Bakterien II. Über die Bindung des Luftstickstoffes durch Azotobacter. Ann. Chem. II, **518**, 109–126.

LINEWEAVER, H. 1938 The Solubility and Chemical and Physical Absorption of Nitrogen Gas in Azotobacter Cells. Jour. Biol. Chem., **122,** 549–567.

LINEWEAVER, H., BURK, D., AND DEMING, W. E. 1934 The Dissociation Constant of Nitrogen-Nitrogenase in Azotobacter. Jour. Amer. Chem. Soc., **56,** 225–230.

PETERS, J. P., AND VAN SLYKE, D. D. 1932 Quantitative Clinical Chemistry. Baltimore, **2,**

ROBERG, M. 1935 Beiträge zur Biologie von Azotobacter. II. Der Stickstoffgehalt der Filtrate von Azotobakterkulturen. Jahrb. f. Wiss. Bot., **82,** 65–98.

ROBERG, M. 1936 Beiträge zur Biologie von Azotobacter. III. Zur Frage eines ausserhalb der Zelle den Stickstoff bindenden Enzyms. Jahrb. f. Wiss. Bot., **83,** 565–592.

WINOGRADSKY, S. 1932 Sur la synthèse de l'ammoniaque par les Azotobacters du sol. Ann. Inst. Pasteur, **48,** 269–301.

BIOGRAPHY

Hans Lineweaver was born December 25, 1907, at Pickens, West Virginia. He graduated from Woodrow Wilson High School, at Beckley, West Virginia, in 1926. He attended Davis and Elkins College, at Elkins, West Virginia, 1926-1929. He was a laboratory assistant for the school year 1928-29. In 1928 he was elected to Chi Beta Phi. He was appointed to the position of Junior Scientific Aide at the Fixed Nitrogen Research Laboratory, U. S. Department of Agriculture, Washington, D. C., August, 1929. He attended George Washington University as a part time student, 1929-1934, and received the A. B. and A. M. degrees respectively in 1930, and 1933. The title of his Master's thesis was "The Characteristics of Oxidation by Azotobacter." He was promoted, by examination, to the position of Junior Chemist at the Fixed Nitrogen Research Laboratory in 1930. He was a Laboratory Assistant at George Washington University, Evening Session, 1931-1932. On leave of absence from the U. S. Department of Agriculture in the academic year 1934-35, he attended Johns Hopkins University as a graduate student in chemistry. He was elected to the Chemists' Club, Phi Lambda Upsilon and to Sigma Xi. He returned to the Department of Agriculture in May, 1935, where the experimental work reported in this thesis was performed. He was promoted to the position of Associate Chemist in the Food Research Division of the Department of Agriculture in April, 1936.